In the reaction catalyzed by GalD, we propose that the active-site base (His^{285}, the homolog of His^{297} in MR) abstracts the 2-R proton of galactonate (Fig. 1C). The immediate product of proton abstraction is an enolic intermediate. This intermediate undergoes vinylogous elimination (*12*) of the 3-hydroxyl group to generate an α,β-unsaturated enol that tautomerizes to the 2-oxo-3-deoxygalactonate product.

With the identification of GalD, we realize that the lysine and histidine general bases found in the active site of MR evolved independently to generate the three different reactions now associated with the MR-MLE-GalD superfamily: racemization catalyzed by MR (requiring both Lys^{166} and His^{297}), β-elimination catalyzed by MLE (with a homolog of Lys^{166} but not of His^{297}), and β-elimination catalyzed by GalD (with a homolog of His^{297} but not of Lys^{166}).

These findings mean that we no longer need to assume that MLE is the immediate evolutionary precursor to MR. Although MR and MLE are both necessary for the catabolism of *R*-mandelate by *P. putida*, the identification of a homolog in carbohydrate metabolism suggests that this superfamily of enzymes may be ubiquitous in nature. In particular, any member of this superfamily of enzymes may have been recruited when the evolution of a metabolic pathway required a reaction in which the α proton of a carboxylic acid must be abstracted (*26*).

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Circadian Clock Mutants in *Arabidopsis* Identified by Luciferase Imaging

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The cycling bioluminescence of *Arabidopsis* plants carrying a firefly luciferase fusion construct was used to identify mutant individuals with aberrant cycling patterns. Both long- and short-period mutants were recovered. A semidominant short-period mutation, timing of *CAB* expression (*toc1*), was mapped to chromosome 5. The *toc1* mutation shortens the period of two distinct circadian rhythms, the expression of chlorophyll *a/b*–binding protein (*CAB*) genes and the movements of primary leaves, although *toc1* mutants do not show extensive pleiotropy for other phenotypes.

Many aspects of plant growth and metabolism are regulated by the circadian clock. Genes that affect circadian rhythmicity have been isolated in *Drosophila melanogaster* (*period* or *per*) and *Neurospora crassa* (*frequency* or *frq*) (*1*). Allelic series at each locus include long- and short-period alleles, whereas the null alleles are largely arrhythmic. Although there is no clear homology of *per* or *frq* sequences to the *Arabidopsis thaliana* genome (*2*), genetic screens for period mutants should nevertheless identify genes required for oscillator function.

A transgenic *Arabidopsis* line was previously constructed in which a fragment of the *Arabidopsis CAB2* promoter mediates circadian-regulated transcription of the firefly luciferase (*Luc*) reporter gene (*3*). We used this *cab2::Ω::Luc* transgenic line to screen for mutant seedlings, in which the first peak of the free-running luminescence cycle occurs either earlier or later than the wild type. M2 seedlings (*4*) were grown for 5 days in 12-hour light:12-hour dark (LD) cycles and then transferred to continuous white light (LL). Candidate mutants were initially selected from M2 populations on the basis of a three-time-point screen (*5*). The mutant candidates were entrained to two LD cycles and assayed again in LL to confirm the mutant phenotype (*6*). We retained 26 "timing of *CAB* expression" (*toc*) lines, which represent at least 21 independent mutations. The mutant phenotypes under LL included seven short-period lines (periods of 21 to 22.5 hours, Fig. 1A), 11 long-period lines (periods of 26 to 28 hours, Fig. 1B), one line with the wild-type period but reduced amplitude (Fig. 1C), and two

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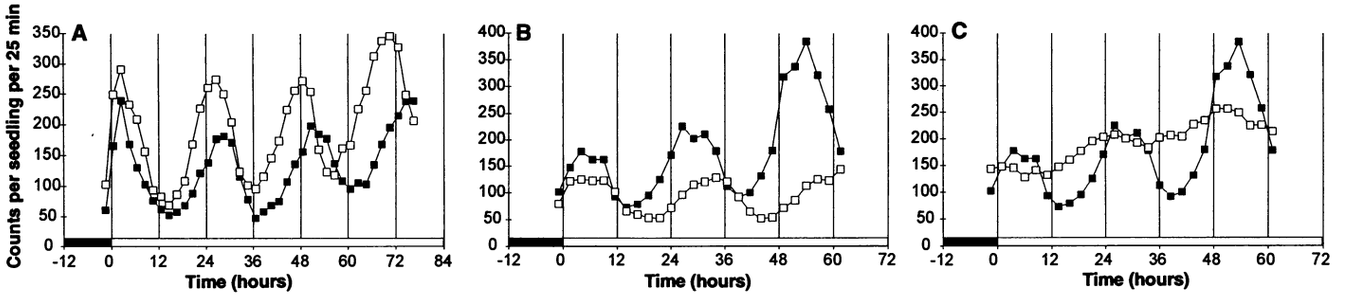
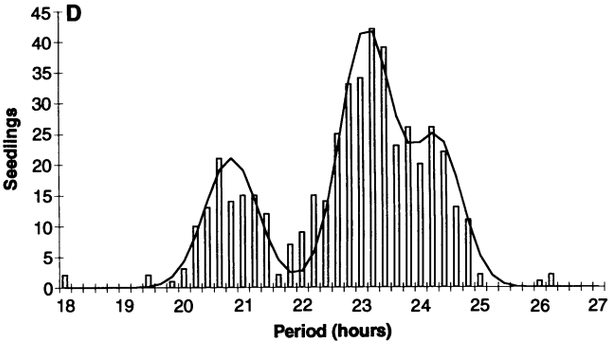


Fig. 1. Luminescence in *toc* mutants. M3 plants were grown for 5 days in LD, transferred to LL, and assayed in the imaging system (*3*). Mean luminescence levels were calculated from data for 25 to 33 single seedlings. (**A**) The *toc1* mutant with a period of ~21 hours. (**B**) A long-period mutant with a period of ~28 hours. (**C**) A low-amplitude mutant with a wild-type period. Filled symbols, wild-type control; open symbols, mutant. Along the abscissa, open box, light period; filled box, dark period. (**D**) The *toc1* period distribution in the F_2 generation. A homozygous mutant seedling from the F_2 of the first back-cross between *toc1* and the transgenic parent was crossed to the Columbia wild type, and 660 seedlings from the F_2 were assayed in LL. Columns represent the distribution of periods. Period bins are labeled with the upper bound. The solid line represents the sum of three normal distributions in a ratio 1:2:1, with the following parameters: 20.7 ± 0.45 hours; 23 ± 0.45 hours; and 24.2 ± 0.4 hours (means ± SD).



lines with the wild-type period but a late phase in LL (*7*). None of the period phenotypes co-segregated with severe morphological phenotypes or with luminescence levels more than twofold different from that of the parent transgenic. The *hy* and *det* mutants, in particular, have distinct morphological phenotypes in light-grown plants (*8*), which were not observed in any of the mutants recovered.

One mutant line (*toc1*) that showed a consistent, short-period phenotype (Fig. 1A) was selected for detailed analysis. The M2 plant was back-crossed to the transgenic parent, as was one of the F_1 progeny of the first back-cross. Self-fertilized *toc1* progeny had a 20.9-hour mean period (SD 0.6 hour) in LL (*9*), approximately 3.5 hours shorter than the parent transgenic line (24.7 ± 0.4 hours) (*10*). The phase of entrainment, amplitude of cycling, and initial luminescence levels were not significantly altered in the mutant (Fig. 1A) (*7*). The *toc1* seedlings grown in light or dark were indistinguishable from the parent transgenic (Fig. 2). The length of the *toc1* hypocotyls (12.7 ± 0.94 mm, n = 25, in DD; 1.06 ± 0.22 mm, n = 25, in LL) was similar to that of the parental line (11.38 ± 1.54 mm, n = 25, in DD; 1.04 ± 0.25 mm, n = 25, in LL) (*11*). Mature light-grown *toc1* plants exhibited no obvious morphological disturbances (*7*); homozygous mutant plants had normal pigmentation, flowered at the same time as wild-type siblings, and were fully fertile.

Segregation of *toc1* away from the transgene indicated that the mutation was independent of the reporter gene. The *toc1* mutant plants from the F_2 generation of a back-cross to the transgenic parent were crossed to the Columbia wild type (*12*). The luciferase-positive class (n = 474) of the F_2 generation (n = 660) was assayed under LL (Fig. 1D). The population contained 16% (106 out of 660) luciferase-positive plants with periods less than 21.4 hours, which is not significantly different (χ^2_1 = 3.1, P = 0.08) from the 19% expected for the segregation of a homozygous mutant class and an unlinked, dominant marker (3 out of 16 possible genotypic combinations). Genomic DNA was prepared from the homozygous mutant plants of this population, and CAPS, RAPD, and SSLP markers (*13*) were used to map *toc1* to the lower arm of chromosome 5 (*14*). The *toc1* gene is on a different chromosome from the transgene and from the *DET1*, *DET2*, and *COP1* genes (*15*). The majority of period mutations in

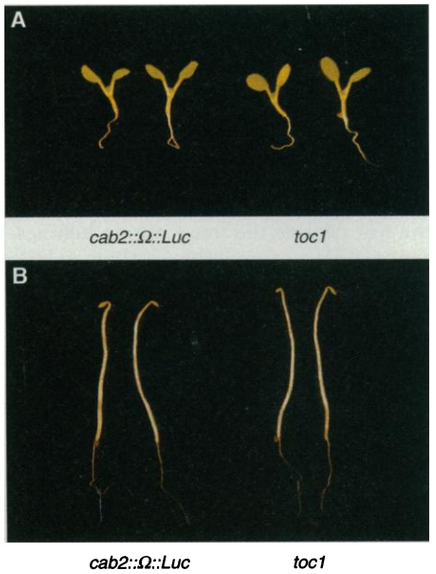


Fig. 2. Morphology of etiolated and light-grown *toc1* plants. Homozygous *toc1* seedlings from the F_3 of the second back-cross to the parental transgenic line and nonmutagenized *cab2::Ω::Luc* controls were grown for 7 days (**A**) in LD or (**B**) in constant darkness. Wild-type plants, *cab2::Ω::Luc*; *toc1* plants, *toc1*.

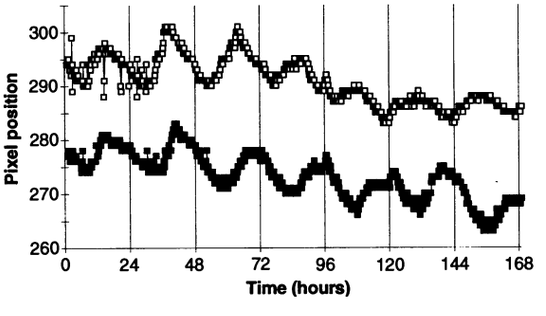


Fig. 3. Leaf movement rhythm in *toc1* plants. *The toc1* seedlings and *cab2::Ω::Luc* controls were grown in LD and transferred to interrupted LL. Leaf positions were recorded in units of screen pixels. Typical traces from one of two similar experiments are shown for one *cab2::Ω::Luc* leaf (filled symbols) and one *toc1* leaf (open symbols).

other organisms are semidominant (*1*), as is *toc1*. The distribution of periods in the large F_2 population of the mapping cross (Fig. 1D) suggests a 1:2:1 segregation with mean periods of 20.7, 23.0, and 24.2 hours, corresponding to *toc1*/*toc1* homozygote, putative *toc1*/*TOC1* heterozygote, and *TOC1*/*TOC1* homozygote classes, respectively.

Clock mutations in other species commonly affect several rhythmic markers in parallel (*1*), suggesting that a single oscillator can control many outputs. *Arabidopsis* exhibits several circadian markers, including rhythms in stomatal aperture (*16*), floral induction (*17*), the expression of nuclear genes (*18*), and the movements of cotyledons and primary leaves (*19*). We used an automated video imaging system to monitor leaf movements in *toc1* plants and the transgenic parent line (*20*). The rhythm of leaf position for typical wild-type and *toc1* leaves is shown in Fig. 3. The leaf movement rhythm had a slightly longer period in the wild type than the rhythm of *cab2::Ω::Luc* luminescence (25.2 ± 1.0 hours, $n = 24$, compared with 24.7 hours for *CAB*). The period of the leaf movement rhythm was significantly shorter in *toc1* plants (23.3 ± 1.3 hours, $n = 18$), indicating that *toc1* encodes a component common to both the *CAB* and leaf movement rhythms.

Mutants in the *det* class are likely to show photomorphogenetic phenotypes and elevated *CAB* expression in dark-grown plants, in addition to their short periods in LL (*8*, *10*). Neither phenotype is found in *toc1* plants. Thus, if *TOC1* is part of the input pathway or pathways defined by *det* and phytochrome mutations, its function must follow a branch point that separates signals to the clock from signals to morphogenesis and to the regulators of *CAB* expression level. Alternatively, *toc1* may affect another input pathway or directly affect the circadian oscillator.

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4. Seed homozygous for the *cab2::Ω::Luc* construct were soaked in 0.4% ethylmethane sulfonate for 12 hours at room temperature and sown in soil (M1 seed). Approximately 4500 M1 plants flowered. Self-fertilized seed (M2 seed) were harvested from pools of approximately 60 M1 plants.
5. The luminescence from 200 M2 seedlings from each of 39 M1 pools (total of ~8000 M2 seedlings) was assayed by video imaging. Images were recorded when luminescence began to rise (19.5 hours after transfer to LL), close to the peak of luminescence (27.5 hours), and again near the trough (37 hours), when luminescence in control seedlings had returned to the level at 19.5 hours. Seedlings were returned to LL after each image.
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The Regulation of Circadian Period by Phototransduction Pathways in *Arabidopsis*

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Transgenic *Arabidopsis* plants expressing a luciferase gene fused to a circadian-regulated promoter exhibited robust rhythms in bioluminescence. The cyclic luminescence has a 24.7-hour period in white light but 30- to 36-hour periods under constant darkness. Either red or blue light shortened the period of the wild type to 25 hours. A phytochrome-deficient mutation lengthened the period in continuous red light but had little effect in continuous blue light, whereas seedlings carrying mutations that activate light-dependent pathways in darkness maintained shorter periods in constant darkness. These results suggest that both phytochrome- and blue light–responsive photoreceptor pathways control the period of the circadian clock.

The fluctuations of light quality and fluence rate in the natural day-night cycle are particularly important to plants, which depend on light as a source of energy, as a developmental signal, and as a cue for tropic movements. Multiple photoreceptors allow the plant to sense the light environment; these include the phytochromes (red–far red receptors), blue light receptors, and at least one ultraviolet-B receptor (*1*). Photoreceptor pathways and circadian systems interact at several levels in higher plants (*2*), though the details of these interactions vary widely among species. The circadian clock has been reported to modulate some photoreceptor functions (*3*). Light pulses or steps control the phase of the clock, mediating entrainment to the day-night cycle (*4*), whereas continuous photoreceptor ac-

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